Genomic Detection of Schmallenberg Virus, Israel

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We discuss genomic detection of Schmallenberg virus in both *Culicoides* midges and affected ruminants during June 2018–December 2019, demonstrating its circulation in Israel. This region is a geographic bridge between 3 continents and may serve as an epidemiologic bridge for potential Schmallenberg virus spread into Asia.

Simbu serogroup viruses form one of the largest serogroups in the genus *Orthobunyavirus* of the family *Peribunyaviridae*, comprising ≥25 antigenically different, but serologically related, negative-sense single-stranded RNA viruses. These viruses are transmitted mainly by *Culicoides* biting midges; they persist in the environment by cycling between infected mammalian hosts and *Culicoides* vectors. Notable examples from the Simbu serogroup are Akabane virus (AKAV), Aino virus, Schmallenberg virus (SBV), Sathuperi virus (SATV), Shamonda virus (SHAV), Peaton virus (PEAV), and Shuni virus (SHUV, which is also suspected of infecting humans.). These viruses are known to cross the placenta of ruminants to the developing fetus, causing abortion, stillbirth, and neonatal malformations that are seen only at birth. The congenital malformations are termed arthrogryposis-hydranencephaly syndrome. Given that the clinical signs can be observed only months after viremia has occurred, field and laboratory practitioners are at a huge disadvantage when facing epidemics caused by these viruses (*1*–*7*).

Until recently, the most studied viruses of the Simbu serogroup were AKAV and Aino virus, both known to be present in Israel (*1*,*3*). In 2011, a new Simbu virus emerged in Europe and was named Schmallenberg virus (SBV) (*8*). Studies suggested that SBV is a reassortant virus, deriving the medium (M) RNA segment from SATV and the small (S) and large (L) RNA segments from SHAV, probably as a result of co-infection of these viruses in either *Culicoides* vectors or the ruminant hosts (*1*,*9*,*10*).

Once SBV emerged in Europe, it was clear to our team in Israel that this virus was either already present in Israel or would be introduced in the future. After AKAV and SHUV outbreaks (*3*,*11*) and virus neutralization test assays showing the additional presence of SATV, SHAV, and PEAV in Israel (*12*), a systematic monitoring system for arboviruses was established in 2015. Serum samples and vectors are collected every month from 13 selected dairy farms representing different geographic regions in Israel (Figure). Specific PEAV, SHUV, and SATV RNA fragments were also detected by nested quantitative PCR (qPCR) from different *Culicoides* species during 2015–2017 (*13*). Furthermore, in 2017, RNA fragments of a specific PEAV were detected in the cerebrospinal fluid (CSF) and testicles of a malformed calf exhibiting hydranencephaly (*14*). SBV was not found in all the studies conducted during 2011–2017, nor was it detected passively in Israel (*3*,*11*–*14*). We report the detection of SBV RNA in Israel in both vectors and affected ruminants.

The Study

During June 2018–December 2019, we trapped 13 pools of *Culicoides imicola*, 8 pools of *C. oxystoma*, 5 pools of *C. puncticollis*, and 5 pools of *C. newsteadii* midges (each pool containing 50 midges) around livestock farms, and we tested CSF from 3 malformed 1-day-old lambs (born on July 3, 2019) and 1 malformed 11-day-old calf (born on November 1, 2019) (Figure; Appendix, https://wwwnc.cdc.gov/EID/article/27/8/20-3705-App1.pdf). We extracted RNA from *Culicoides* homogenates and CSF using Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega, https://www.promega.com) according to the manufacturer's instructions. We used total viral nucleic acids (0.4 μg) for cDNA synthesis

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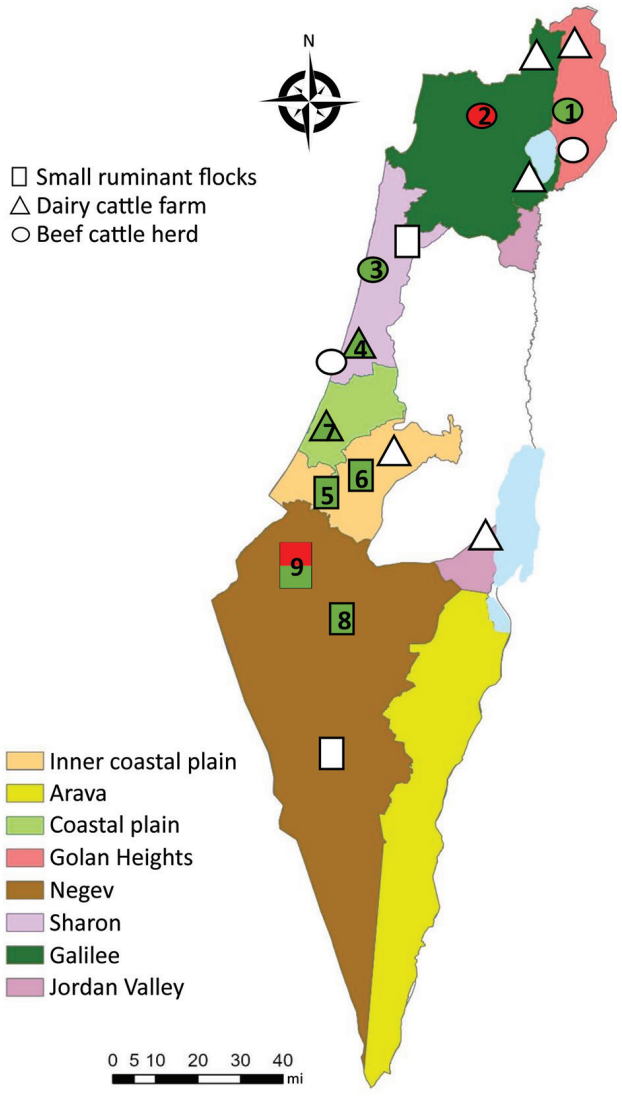

Figure. Locations and types of farms sampled in study of Schmallenberg virus (SBV), Israel. Farm numbers match those listed in Table 2. Green, farms from which SBV-positive *Culicoides* pools were collected; red, farms on which SBV-positive malformed progeny were detected.

by UltraScript Reverse transcription (PCR Biosystems, https://www.promega.com) according to the manufacturer's instructions. We performed reverse transcription (RT) nested qPCR targeting the L RNA segment of Simbu serogroup viruses according to Behar et al. (*13*). We further subjected samples suspected of being Simbu serogroup positive to RT-nested and seminested PCRs performed using S, M, and L segment-specific primer sets (Table 1; Appendix).

Of the 31 species-specific pools from the 4 *Culicoides* midge species that are known or suspected to be vectors of Simbu serogroup viruses (i.e., *C. imicola*, *C. oxystoma*, *C. puncticollis*, *C. newsteadii*) (*2*,*12*,*15*), we found that 11 contained RNA of Simbu serogroup viruses in 2018 and 2019 (35% of the total pools tested) (Table 2; Figure; Appendix Table). We identified partial nucleotide sequences of the S (370/830 bp) and L (370/6,882 bp) segments. Phylogenetic analysis of the samples showed that all positive samples were virtually identical to SBV (GenBank accession nos. MT816474–82, MT816485–95) (Appendix Figure, panels A, C). These samples were collected from several different geographic regions in Israel (Table 2, lines 1 and 3–12 in the Samples column; Figure; Appendix Table). In addition, we detected SBV RNA-specific fragments of the S (370/850 bp), M (430/4,373 bp), and L segments (370/6,882 bp) in a CSF sample from a malformed lamb born in July 2019 on a farm in southern Israel (Negev desert) and a malformed calf born in November 2019 on a farm in northern Israel (Galilee) (GenBank accession nos. MT816472, MT816473, MT816483, MT816484, MT816496, MT816497) (Table 2, lines 2 and 13 in the Samples column; Appendix Figure).

In general, the most susceptible period for induction of congenital malformations by Simbu serogroup viruses is 65–70 days of gestation in lambs and 150 days of gestation in calves (*1*,*7*). Thus, SBV detection

Table 1. Primer sets used for the amplification of Schmallenberg virus RNA-specific fragments of the S, medium M, and L segments by reverse transcription nested PCR*

Segment	External primer sequence, 5′ → 3′	Internal primer sequence, 5′ → 3′	Expected product size, bp	Reference
S	AKAI206F: CAC AAC CAA GTG TCG ATC TTA SimbuS637: GAG AAT CCA GAT TTA GCC CA	S_nestF: TGG TTA ATA ACC ATT TTC CCC A S_nestR: GTC ATC CAY TST TCW GCA GTC A	370	External: (*4*); internal: this study
M	924F: CCG AAA ACA AGG AAA TTG TG 2331R: GGT TCA AAC ATC TCT AGG C	1899F: TAT AGT CCC TGG ATT AGG TC 2331R: GGT TCA AAC ATC TCT AGG C	430	Forward primers: (*8*); reverse primers: this study
L	SNL_F: GCA AAC CCA GAA TTT GYW GA SNL_R: ATT SCC TTG NAR CCA RTT YC	panOBV-L-2959 F: TTG GAG ART ATG ARG CTA ARA TGT G panOBV-L-3274R: TGA GCA CTC CAT TTN GAC ATR TC	370	External: this study; internal: (6)

*L, large; M, medium; S, small.

Table 2. Samples that tested positive for Schmallenberg virus by reverse transcription nested PCR, Israel*

Geographic region	Sample source	Collection date	Infected farm type (farm no.)
Golan Heights (latitude 34.1)	*Culicoides oxystoma* midge	2018 Sep	Beef cattle (1)
Galilee (latitude 32.7–33.5)	Malformed calf	2019 Nov	Beef cattle (2)†
Sharon plain (latitude 32.2)	*C. imicola* midge‡	2018 Jun	Beef cattle (3)§
	C. puncticollis midge	2018 Jun	Beef cattle (3)§
	C. newsteadii midge	2018 Jun	Beef cattle (3)§
	C. imicola midge	2018 Jul	Dairy cattle (4)
Interior plain (latitude 31.89)	*C. imicola* midge‡	2018 Nov	Small ruminant farm (5)†§
	C. imicola midge	2018 Nov	Small ruminant farm (5)†§
	C. imicola midge‡	2019 Dec	Small ruminant farm (6)§
Coastal plain (latitude 31.89)	*C. oxystoma* midge	2018 Jun	Dairy cattle (7)
Negev desert (latitude 29.7–30.714)	*C. oxystoma* midge	2018 Nov	Small ruminant farm (8)§
	C. puncticollis midge	2019 Jul	Small ruminant farm (9)†¶
	Malformed lamb	2019 Jul	Small ruminant farm (9)†¶
South Jordan Valley (latitude 31.56)	NA	NA	NA

*NA, not applicable.
†Farms on which dams and ewes gave birth to stillborn and malformed neonates.
‡Samples were confirmed positive at Friedrich Loeffler Institute, Greifswald, Germany.
§Farms expecting a rate of 80%–85% prolificacy, but during calving season showed only 50%–65% prolificacy.
¶Sheep farm from which both insects and malformed lambs were sampled.

in the respective ruminants fits with viral infection in March–April 2019, suggesting exposure to SBV in Israel in early spring 2019. Nevertheless, reports on severe decline in progeny prolificacy, stillbirths, and malformed lambs were reported by farmers to the Veterinary Field Services from autumn 2018 through December 2019 (Table 2). The detection of SBV in *Culicoides* pools collected from several of those farms (Table 2, lines 3–5, 7–9, and 11–12 in the Sample column; Figure; Appendix Table) suggests that SBV might have been clinically affecting ruminants in Israel as early as June 2018.

Conclusions

Our results demonstrate the circulation of SBV outside Europe. Future studies are needed to determine the seroprevalence of SBV in the Middle East, because this information is essential for understanding the risk of SBV spread into countries in Asia. Because SATV is found in the Middle East (*12*,*13*), virus neutralization tests will probably not be able to properly distinguish between antibodies against SBV and those against SATV. Therefore, developing a competitive ELISA system using SBV-specific antibodies is crucial. Finally, the presence of both SATV and SBV in Israel provides a unique opportunity for comparative studies on possible cross-protection of SBV commercial vaccines between these viruses.

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Appendix

Materials and Methods

Culicoides Collection

Culicoides midges were trapped on livestock farms from 7 geographic regions in Israel (Figure, main article): Negev desert (latitude 29.7–30.714086; 3 small ruminant farms), South Jordan valley (latitude 31.56; 1 dairy farm), interior plain (latitude 31.89; 1 dairy farm, 2 small ruminant farms), coastal plain (latitude 31.89; 2 dairy farms), Sharon plain (latitude 32.2; 1 dairy farm, 1 beef cattle farm, 1 small ruminant farm), Galilee (including the north Jordan valley; latitude 32.7–33.5; 2 dairy farms, 1 beef cattle farm), and Golan Heights (latitude 34.1; 1 dairy farm, 2 beef cattle farms) during June 2018–December 2019.

Culicoides midges were collected using suction light traps equipped with an 8 W blacklight and a downdraft suction motor powered by 2 rechargeable 1.5 V GP2700 AA batteries. Insects were collected into a reusable plastic jar suspended below the trap's fan. Two light traps were placed overnight (1 h before sunset and retrieved 1 h after dawn) at suitable locations on each farm, as close to the livestock as possible, suspended at a height of 1.7–2 m above the ground. Immediately after collection, the plastic jars containing live *Culicoides* midges were placed in 4°C cooling boxes and transported to the laboratory. Upon their arrival, the live insects were anesthetized with CO_2 and sorted to species under a stereoscopic microscope (Nikon SMZ25, https://www.microscope.healthcare.nikon.com) using various taxonomic keys (*1*–*4*). After sorting, live *Culicoides* midges were grouped in pools according to location and species, and all pools were stored at −80°C until testing for the presence of Simbu serogroup viruses. In total, 13 pools of *C. imicola*, 8 pools of *C. oxystoma*, 5 pools of *C. puncticollis*, and 5 pools of *C. newsteadii* (each pool contained 50 midges) were analyzed (Table 2, main article). Pooled *Culicoides* were homogenized according to Behar et al. (*5*).

Genomic Detection of SBV

A 200-μL aliquot of *Culicoides* homogenate or cerebrospinal fluid was used for total viral nucleic acid extraction with the Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega, https://www.promega.com) according to the manufacturer's instructions. The remaining 300 μL was kept at −80°C for later use. Total viral nucleic acids (0.4 μg) were used for cDNA synthesis by UltraScript Reverse transcription (PCR Biosystems, https://pcrbio.com) according to the manufacturer's instructions. cDNA synthesis and subsequent initial RT-qPCR amplifications targeting the large segments of Simbu serogroup viruses were performed using Pan Simbu primers according to Fischer et al. (*6*). However, during our work with this system, our internal quality control indicated that we got a higher frequency of false positives because of a nonspecific amplification of *Culicoides* ribosome (18s rRNA) from the *Culicoides* homogenates. Moreover, we discovered that *Culicoides* homogenates have a high inhibitory effect. Consequently, a second amplification step was added to provide higher specify to our detection systems and RT-nested qPCR was performed according to Behar et al. (*5*). All samples were run in duplicate. Reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, https://www.bio-rad.com) with the manufacturer-recommended PCR parameters. Samples with a cycle threshold (C_t) value <30 and melting temperature (T_m) of 73°C–81°C were suspected of being Simbu serogroup positive. For further and more accurate analysis, nested and semi nested PCRs were performed as follows.

Pan Simbu RT-PCR targeting the S RNA segment was conducted according to Hirashima et al. (*7*). The PCR products served as the template for the nested PCR with primer pairs Simbu_S_nestF and Simbu_S_nestR (Table 1) (expected product size ca. 370 bp).

Pan Simbu RT-PCR targeting the large (L) RNA segments was conducted using external primers SNL_F and SNL_R (Table 1 in main article). The PCR products served as the template for the nested PCR according to Fischer et al. (*6*) (expected product size ≈370 bp).

RT-PCR targeting the medium (M) segment specific to SBV was conducted using primers 924F according to McGowan et al. (*8*) and 2331R: 5′-GGTTCAAACATCTCTAGGC-3′. The PCR products served as the template for the semi-nested PCR using primer 1899F according to McGowan et al. (*8*) and 2331R (expected product size ≈430 bp).

Negative controls (no DNA added) were always performed in parallel. No products were obtained from these controls. Positive controls were added only to the final nested or semi-nested step to avoid contamination. All samples were amplified in a conventional PCR (SensoQuest Labcycler, https://www.sensoquest.de) with 1 μM primer and addition of 10 ng/μL bovine serum albumin (BSA) in 2X PCRBIO HS Taq mix (PCR Biosystems) for a total volume of 25 μL reaction mixture according to the manufacturer's instructions. BSA was added to improve PCR amplification because the amounts of viral nucleic acid extracted from *Culicoides* and ruminant samples were relatively low. Products were separated on a 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and stained with SmartGlow PS (Accuris Instruments, https://www.accuris-usa.com). Negative controls (no template added) were always run in parallel. No products were obtained from these controls. All PCR products were then sequenced in both directions. Sequence chromatograms were visually inspected, verified, aligned, and annotated using Geneious Pro (Biomatters, https://www.geneious.com). Phylogenetic analysis was performed using maximum likelihood implemented in PhyML (*9*). To assess confidence in the nodes, 100 bootstrap replicates were performed. All sequences were selected for phylogenetic analysis and were deposited in GenBank under accession nos. MT816472–MT816497.

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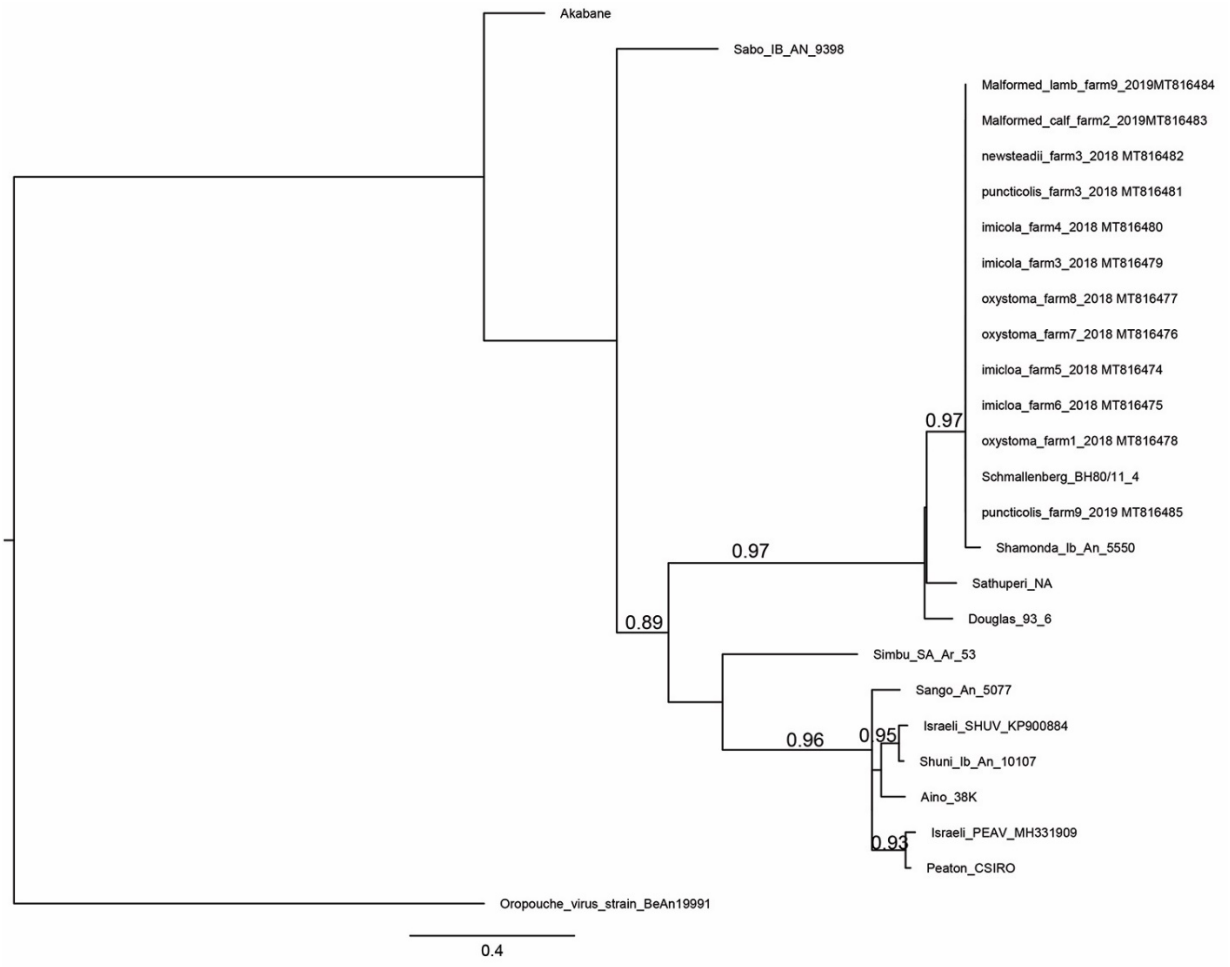
Appendix Table. Viral genomic detection in field-collected *Culicoides* species in 2018 and 2019, Israel*

Geographical region	Date	Farm type	*Culicoides imicola*		*Culicoides oxystoma*		*Culicoides puncticollis*		*Culicoides newsteadii*	
			(n/+)	Virus genome detected	(n/+)	Virus genome detected	(n/+)	Virus genome detected	(n/+)	Virus genome detected
Golan Heights	2018 Sep	Beef cattle (1)†	0	NA	1/1	SBV	0	NA	0	NA
	2019 Sep	Beef cattle	0	NA	1/0	NA	0	NA	0	NA
Galilee	2018 Sep	Dairy	0	NA	1/0	NA	0	NA	0	NA
	2019 Oct	Dairy	1/0	NA	0	NA	0	NA	0	NA
Sharon plain	2018 Mar	Small ruminant	0	NA	0	NA	0	NA	1/0	NA
	2018 Jun	Beef cattle	1/0	NA	1/0	NA	0	NA	0	NA
	2018 Oct	Beef cattle (3)	1/1	SBV	1/0	NA	1/1	SBV	1/1	SBV
	2018 Jul	Dairy (4)	1/1	SBV	1/0	NA	1/0	NA	0	NA
	2019 Nov	Beef cattle (3)	2/2	Aino, AKAV	0	NA	1/1	Aino	0	NA
Interior plain	2018 Nov	Small ruminant (5)	2/2	SBV	0	NA	0	NA	0	NA
	2018 Oct	Dairy	0	NA	0	NA	0	NA	1/0	NA
	2019 Sep		0	NA	0	NA	0	NA	1/0	NA
	2019 Dec	Small ruminant (6)	0	NA	0	NA	0	NA	0	NA
Coastal plain	2018 Jun	Dairy (7)	1/0	NA	1/1	SBV	0	NA	0	NA
South Jordan valley	2018 Mar	Dairy	1/0	NA	0	NA	0	NA	0	NA
	2019 Sep		0	NA	0	NA	1/0	NA	0	NA
Negev	2018 Nov	Small ruminant	1/1	SBV	0	NA	0	NA	1/0	NA
	2018 Nov	Small ruminant (8)	1/1	SBV	1/0	NA	0	NA	0	NA
	2019 Jul	Small ruminant (9)	1/0	NA	0	NA	1/1	SBV	0	NA

*Aino, Aino virus; AKAV, Akabane virus; NA, not applicable; SBV, Schmallenberg virus.
†Numbers refer to affected farms, related to those in Table 2 and Figure, main article. (In farm 2, *Culicoides* were not collected. Only ruminant samples were collected.)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Douglas_93_6
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Simbu_SA_Ar_53
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0.95
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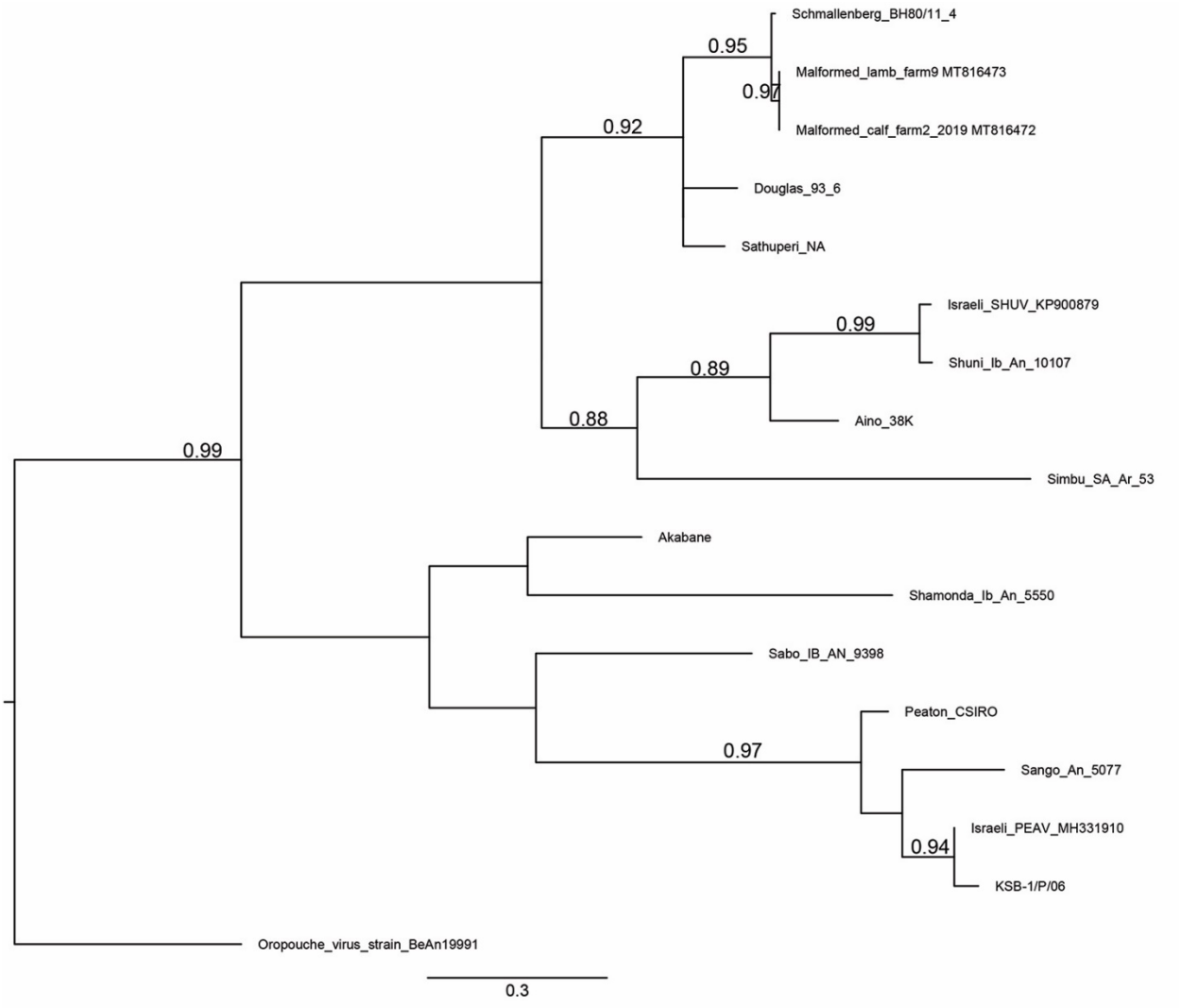
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Sathuperi_NA
Israeli_SHUV_KP900879
0.99
Shuni_Ib_An_10107
0.89
Aino_38K
0.88
0.99
Simbu_SA_Ar_53
Akabane
Shamonda_Ib_An_5550
Sabo_IB_AN_9398
Peaton_CSIRO
0.97
Sango_An_5077
Israeli_PEAV_MH331910
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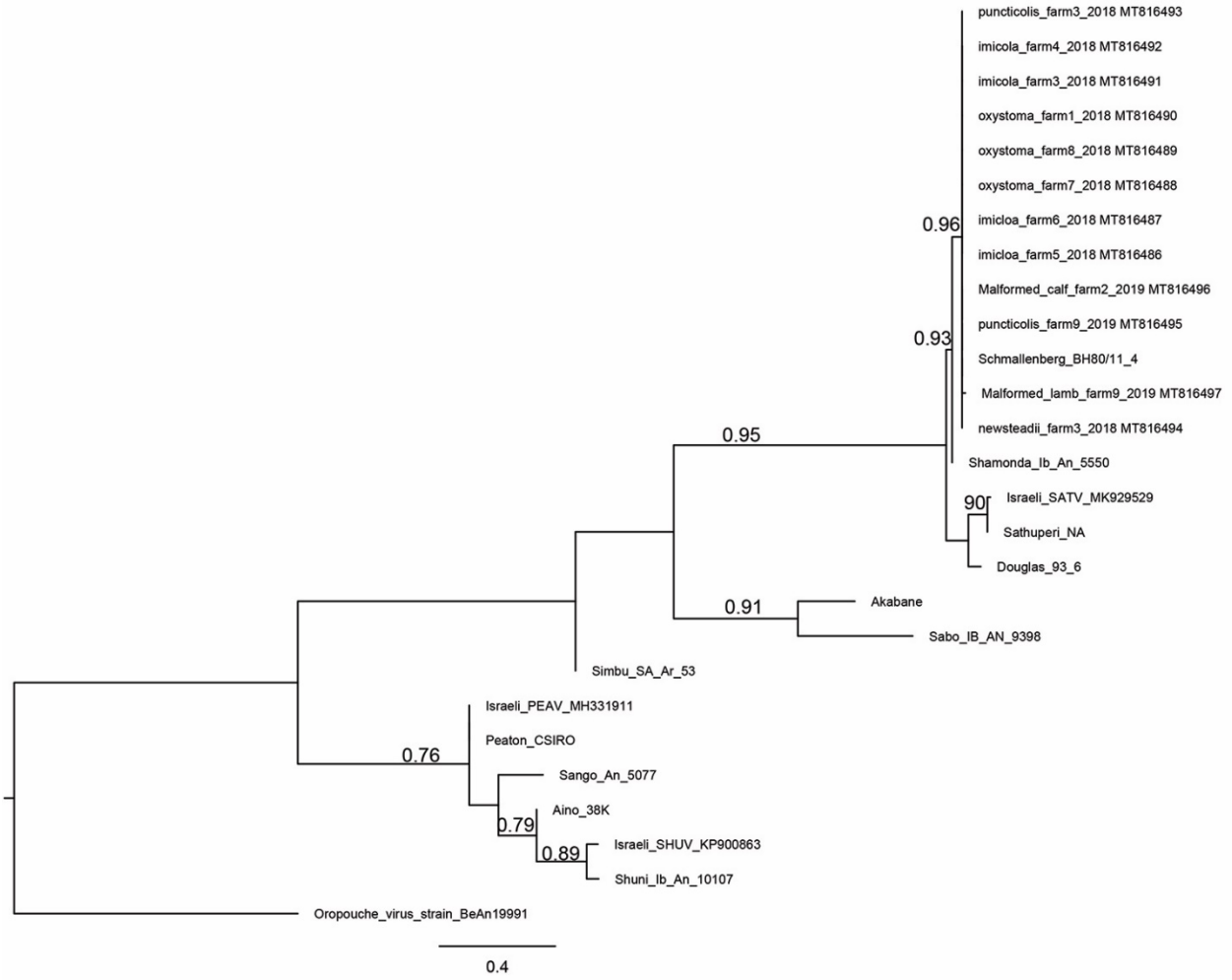
KSB-1/P/06
Oropouche_virus_strain_BeAn19991
0.3



Appendix Figure. Rooted maximum-likelihood phylogenetic tree of Simbu serogroup for the (A) S, (B) M, and (C) L segments, based on a general time-reversible and gamma-distributed rate heterogeneity (GTR_G) model of nucleotide substitution. Sequences of SBV detected in positive pools of *C. imicola*, *C. oxystoma, C. newsteadii*, and *C. puncticollis* collected from different geographic regions in Israel were compared with previously published sequences of specific Peaton virus (PEAV), Shuni virus (SHUV), and Sathuperi virus (SATV) RNA fragments obtained in Israel during 2014–2017, and with the respective validated whole-genome sequences of Simbu serogroup viruses. Whenever possible, we used Simbu serogroup viruses for which full-segment sequences were available. Homologous sequences from Oropouche virus were used as the outgroup. Scale bar indicates estimated nucleotide substitutions. Only bootstrap values greater than 70% are shown.